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Infrared Spectroscopy in Clinical and Diagnostic Analysis

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The infrared spectrum of a mixture serves as the basis to quantitate its constituents, and a number of common clinical chemistry tests have proven to be feasible using this approach. This article reviews the infrared spectroscopy-based analytical methods that have been developed for consideration as clinical assays, including serum analysis, urine analysis, amniotic fluid assays for the estimation of fetal lung maturity, and others. Because of the widespread interest in the potential for in vivo measurement of blood glucose using near-infrared spectroscopy, a separate section is devoted to the analysis of glucose in whole blood.

A related technique uses the infrared spectrum of biomedical specimens directly as a diagnostic tool. For example, the spectra of serum and of synovial fluid have proven to be useful in the diagnosis of metabolic disorders and arthritis, respectively, without explicitly recovering their chemical composition from the spectra. Rather, characteristic spectral features and patterns have been identified as the basis to distinguish spectra corresponding to healthy patients from those corresponding to diseased patients. These applications are reviewed here.

Issues such as ease of use, speed, reliability, sample size, and calibration stability all play important roles in governing the practical acceptability of infrared spectroscopy-based analytical methods. To provide a framework to illustrate these issues, descriptions are included for the various procedures that have been explored to wed successfully infrared spectroscopy to clinical chemistry.

1 INTRODUCTION

Infrared (IR) spectroscopy has emerged in recent years as the analytical method of choice in an enormous variety of applications. What brought about this revolution? The clearest advantage is that no specific reagents are required. Automated, repetitive analyses can therefore be carried out at very low cost. The appeal of these factors has spurred the development of a new generation of analytical IR spectrometers that combine high acquisition speed with superb spectral sensitivity. Powerful chemometric algorithms and software packages have emerged in parallel with the new hardware, and new applications emerge continually.

Rather than relying upon reagents to promote color reactions, IR-based analysis is founded upon the spectrum of IR colors characteristic of the analyte itself. If a particular component provides an IR absorption spectrum, and its concentration is high enough that the spectrum contributes meaningfully to the IR absorption profile, then it may, in principle, be quantified by using IR spectroscopy. Although the requirement that the

component exhibits an IR absorption spectrum rules out the quantitation of simple ions, a number of very common clinical analytical tests may, in principle, be carried out using IR spectroscopy.

This article begins by comparing and contrasting mid-infrared (MIR) and near-infrared (NIR) spectroscopy in the context of analytical applications. The second section describes the general approach to generating an IR-based quantitation method. Although Beer's law generally holds true for common analytes in biological fluids, it is very unusual to find a single absorption that can be used as the basis to quantify any single component in real-life samples. Analytical methods that are based upon IR spectroscopy must nearly always be calibrated by reference to accepted clinical analyses, using multiple-wavelength linear regression or other full-spectrum methods.

The function of the clinical chemistry laboratory is to perform quantitative and qualitative analyses on body fluids such as serum, blood, urine, and spinal fluid, as well as other materials such as tissue, calculi, and feces. The main body of this article describes IR-based methods to carry out some of the most common clinical analytical tests, specifically those involving serum, whole blood, and urine. Fluids that are less commonly assayed (e.g. saliva and amniotic fluid) are also discussed separately. NIR spectroscopy has achieved some notoriety in the clinical chemistry arena because of the early promise that it might serve as the basis for a noninvasive blood glucose test. Some relevant *in vitro* studies are surveyed briefly here. The article closes with a discussion of novel approaches to derive diagnosis directly, without explicit quantitative analysis, from the spectra of biological fluids.

2 INFRARED SPECTROSCOPY OF BIOLOGICAL FLUIDS

The IR spectral region ranges from the red end of the visible spectrum at 780 nm ($12\,820\text{ cm}^{-1}$) to the onset of the microwave region at a wavelength of 1 mm (10 cm^{-1}). Traditionally, this range is further subdivided into the near-infrared (NIR), mid-infrared (MIR), and far-infrared (FIR). The MIR region covers the range $400\text{--}4000\text{ cm}^{-1}$, and is the region most familiar to the organic chemist as providing a "fingerprint" characteristic of molecular species. It is this region that includes the rich spectrum of absorptions corresponding to fundamental vibrations of the species being probed.

Although MIR absorption positions are almost universally reported in units of wavenumbers (cm^{-1} ; the inverse of the wavelength in centimeters), it remains the norm for NIR spectra to be reported in wavelength

units, generally in nanometers. The NIR spans the range 780–2500 nm, encompassing weak transitions that correspond to combinations and overtones of the vibrational modes observed in the MIR. Because NIR absorptions are generally broad and therefore strongly overlapping, it is difficult or impossible to arrive at specific assignments for individual absorptions in the NIR. Partly for that reason, these transitions were long ignored by the spectroscopic communities (indeed by all communities!), although their potential for use in analytical work was noted as early as the mid-1950s.⁽¹⁾ By the end of the following decade, the technique had caught the attention of the agricultural community as a possible means to determine protein, oil, and moisture content of agricultural commodities. In this application and many others developed since, the inherently weak absorptions proved advantageous, permitting the convenience of longer optical path lengths than are feasible in MIR work, and hence relatively easy sample handling. Since the seminal work of Norris,⁽²⁾ Williams,⁽³⁾ and others, NIR spectroscopy has largely matured, and now finds acceptance in an enormous variety of analytical applications. The majority of NIR spectrometers manufactured today are customized for analytical applications, including appropriate software and simplified user interfaces for routine operation.

In considering the use of IR spectroscopy for clinical analyses, we are confronted with the fact that the most abundant species found in all biological fluids is water, and the IR spectra reflect this fact. To illustrate the dominance of water in the IR spectra, Figures 1 and 2 depict the absorption profiles for native serum in the MIR and NIR spectral regions. Although some of the stronger solute absorptions do emerge in the MIR spectra, water clearly dominates the overall appearance. The NIR spectra are

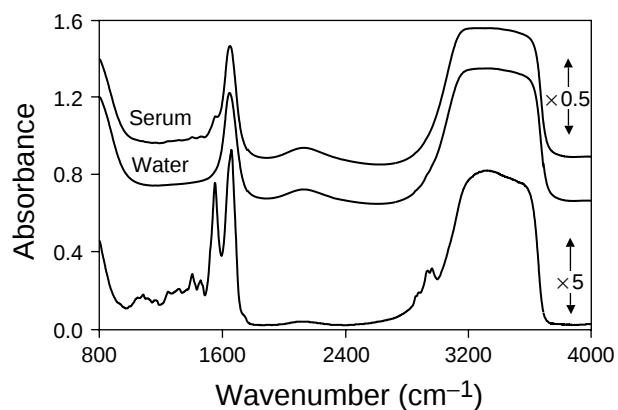


Figure 1 MIR absorption spectra of serum and of water, collected with an optical path length of $6\text{ }\mu\text{m}$. The lower trace is a difference spectrum, with the spectrum of water subtracted from that of serum. Note the tenfold difference in the absorbance scales.

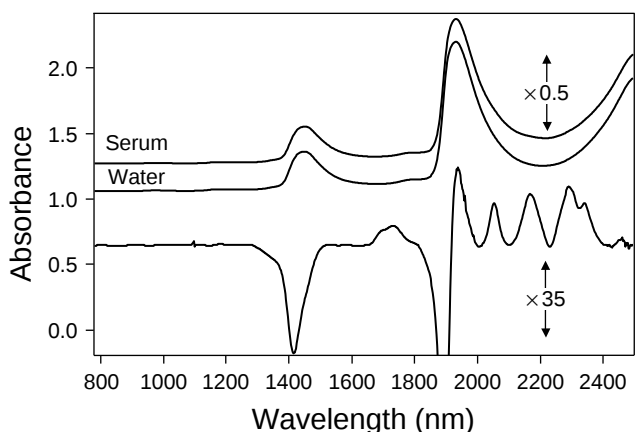


Figure 2 NIR absorption spectra of serum and of water, collected with an optical path length of 0.5 mm. The lower trace is a difference spectrum, with the spectrum of water subtracted from that of serum. Note the 70-fold difference in the absorbance scales.

apparently devoid of any absorptions other than those of water.

MIR and NIR spectroscopies in fact offer quite different, complementary, approaches to analysis. The richness of the MIR spectrum makes it instinctively appealing as the method of choice for analytical work, however NIR has practical benefits such as convenience in sample handling and the fact that the sample cells do not require specialized materials. Whereas MIR spectroscopy of aqueous specimens typically requires optical path lengths of the order of microns, NIR transmission spectra are generally collected using path lengths of 0.5 mm or greater. The question of whether to use NIR or MIR spectroscopy for analytical purposes then translates to the question of whether the additional effort generally required to acquire MIR spectra is compensated by other possible benefits such as greater analytical accuracy or smaller sample volume.

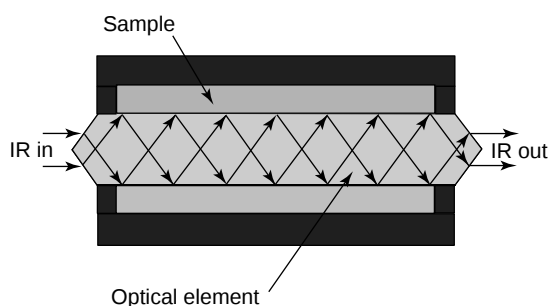


Figure 3 Apparatus to measure the ATR spectrum for a liquid specimen. The ATR spectrum is derived by ratioing the single-beam spectrum measured with the specimen in place against a single-beam spectrum for the clean optical element.

2.1 Mid-infrared Attenuated Total Reflectance Spectroscopy

Attenuated total reflectance (ATR) spectroscopy provides an alternative means to measure absorption spectra by using the experimental arrangement illustrated in Figure 3. The clearest advantage of this method is that it provides a means to measure MIR spectra for strongly absorbing aqueous solutions, without the inconvenience and imprecision involved in working at very short path lengths that are required for transmission spectroscopy. Rather than transmitting IR radiation through the specimen, the liquid sample is placed in contact with the ATR optical element.^(4,5) The refractive index of the element (typically zinc selenide) is high enough that the IR beam propagating through it undergoes several internal reflections as it travels through the crystal. A background spectrum is first measured with no sample in the cell. The sample is then placed in contact with the crystal. The internally reflected beam effectively penetrates the sample to depths of 0.5–2 μm and hence is attenuated at wavelengths corresponding to sample absorptions. Ratioing the resulting single-beam spectrum against the background spectrum results in a spectrum that is nearly identical to the absorption spectrum, differing only by virtue of the wavelength dependence of the penetration depth.

2.2 Mid-infrared Spectroscopy of Dried Films

This approach finesses the difficulties associated with strong water absorptions by simply eliminating water from the specimen. Typically 5–50 μL of liquid is spread on a suitable substrate and allowed to dry, and a transmission spectrum is acquired for the resulting film. In addition to eliminating the spectral interference of water,

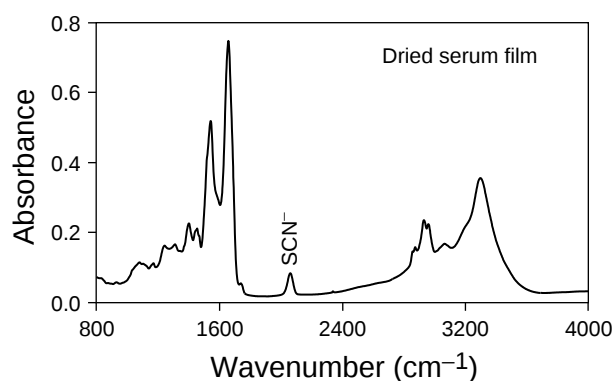


Figure 4 Absorption (transmission) spectrum for a serum film dried onto a barium fluoride window. The specimen was first diluted twofold in aqueous 4 g L⁻¹ potassium thiocyanate (KSCN) solution. The absorption of SCN⁻ at 2060 cm⁻¹ was used for subsequent normalization of the spectra as part of the development of quantitation models (see Shaw et al.⁽²³⁾).

this approach can provide inherently better spectral resolution by virtue of eliminating the water/solute interactions. A representative spectrum of a dry serum film is illustrated in Figure 4.

2.3 Near-infrared Spectroscopy

Although the NIR is defined as encompassing the 780–2500 nm spectral range, it is convenient to subdivide further this span into natural subregions. The 2000–2500 nm range includes the most intense absorptions and thus is the region most commonly exploited for analytical purposes. Absorptions in this region correspond to “combination bands”, combining X–H (where X = C, N, O) stretches with other fundamental vibrations, whereas practically all of the higher energy transitions correspond to vibrational first (1400–1800 nm), second (950–1250 nm), and third overtones (Table 1).

The diversity of transitions in the NIR region has interesting practical consequences. For aqueous solutions, the 2000–2500 nm region is best explored by using a transmission cell with an optical path length of 0.5–2.5 mm. The optimal path length to observe the first overtone transitions is longer – of the order of 5–10 mm – whereas observation of the second overtones requires a path length of several centimeters. Where sample volume is a consideration, a relatively short path length is a necessity and the combination region is therefore preferred. Another outgrowth of this trend is that tissue is relatively transparent at shorter NIR wavelengths. A key consideration in the search for in vivo analytical methods (e.g. blood glucose) is therefore to arrange the experiment such that the effective optical path length is optimized for the appropriate analyte NIR absorption features. A proposed in vivo method based upon the combination bands will require a short effective path length, whereas a method that monitors second overtone absorptions would require a much longer one.

Table 1 NIR vibrational transitions

NIR spectral range (nm)	Nature of vibrational transitions
2200–2450	C–H stretch combinations
2000–2200	N–H, O–H stretch combinations
1650–1800	C–H stretch, 1st overtones
1400–1500	N–H, O–H stretch, 1st overtones
1100–1225	C–H stretch, 2nd overtones
950–1100	N–H, O–H stretch, 2nd overtones
850–950	C–H stretch, 3rd overtones
775–850	N–H stretch, 3rd overtones

3 CALIBRATION METHODS

In the vast majority of cases, IR-based analytical methods are developed via calibration to accepted reference analyses. The term “calibration” therefore describes the derivation of a model with which to recover quantitative analytical information from the IR spectra. Although this step is obviously a trivial one for very simple one- or two-component systems, more complex mixtures require a more sophisticated approach.

The general procedure is the same regardless of the details of the process. The first stage is to accumulate both IR spectra and reference assays for a set of appropriate clinical specimens. Ideally, this set of calibration samples should span the range of concentrations expected both for the analyte of interest and for any interfering species (i.e. any IR absorber other than the target compound). Separate calibration models are then developed for each of the target analytes. Finally, each of the calibration models is validated by comparing IR-predicted levels to the reference levels determined for an independent set of test specimens. An outline of the model development process is presented in Table 2.

This section introduces three of the more common techniques: multiple-wavelength linear regression (MLR),

Table 2 Development of an IR-based clinical analytical method

<i>Preparation</i>
• Collect clinical specimens • Carry out reference analyses for species of interest • Measure corresponding IR spectra • Designate two-thirds of the total number of spectra as the calibration set and the remaining one-third of spectra as the validation set • Preprocess spectra
<i>Calibration</i>
• Choose modeling method (e.g. MLR, PLS, PCR) • Generate models ranging in complexity from a very few variables (wavelength terms for MLR; factors for PLS, PCR) to many variables • Predict concentrations using all models and compare to reference analyses • Identify outliers and correct or remove as appropriate • Recalibrate models with outliers removed • Evaluate standard errors of calibration and cross-validation for each model and plot as a function of the number of variables in the model
<i>Validation</i>
• Predict concentrations using all models and compare to reference analyses • Evaluate standard error of prediction for each model and plot as a function of the number of variables in the model

Where IR methods are sought for more than one species, the calibration/validation procedure is carried out independently for each analyte.

principal component regression (PCR), and partial least squares (PLS).

3.1 Multiple-wavelength Linear Regression

This approach is simply an extension of Beer's law to include multiple wavelengths. The need for several wavelengths is dictated by the inherent richness of the IR spectra – it is generally difficult or impossible to find a single absorption corresponding to a particular analyte that is not overlapped by the absorptions of at least one other constituent. The Beer's law relationship shown in Equation (1):

$$A = \varepsilon cL \quad (1)$$

is therefore replaced by the more general form shown in Equation (2):

$$A = \sum \varepsilon_i c_i L \quad (2)$$

where A is the absorbance, ε_i is the molar absorptivity of the i th constituent, c_i is the concentration of the i th constituent, and L is the optical path length. The expression relating the concentration to IR absorption intensities then takes the form of Equation (3):

$$c_i = K_{0i} + K_{1i}A(\lambda_1) + K_{2i}A(\lambda_2) + \cdots + K_{Ni}A(\lambda_N) \quad (3)$$

where K_{Ni} are the calibration coefficients for the i th constituent, and λ_N are the corresponding analytical wavelengths.

This approach is most readily applied when the spectra show dominant absorptions corresponding to the analyte of interest. This proved to be the case in the NIR analysis of urine urea.⁽⁶⁾ Figure 5 compares the spectra of five representative urine specimens in

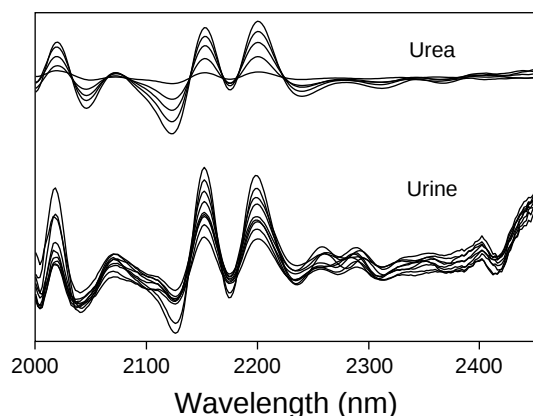


Figure 5 NIR spectra (second derivatives, inverted) for a representative set of nine urine specimens (lower traces) and for five aqueous urea samples ranging from 30 to 230 mmol L⁻¹ (upper traces).

the NIR region of 2050–2500 nm, together with the spectra of five aqueous urea solutions spanning the concentration range 30–230 mmol L⁻¹. In this instance the spectral features due to urea clearly dominate the urine spectra, and it is not surprising that a single-wavelength Beer's law relationship provided quite good accuracy in extracting the urea concentrations. In particular, the intensity of the feature at 2152 nm proved sufficient to recover urea concentrations with a standard error of 20 mmol L⁻¹ over the physiological concentration range of 100–500 mmol L⁻¹. The accuracy was improved, however, by including additional terms as shown in Equation (4):

$$C(\text{urea}) = -10 + 68 \left[\frac{A(2152 \text{ nm})}{A(1194 \text{ nm})} \right] + 1.3 \times 10^5 A(1724 \text{ nm}) \quad (4)$$

There are two new wavelengths in this model. The first, at 1194 nm, corresponds to a weak water absorption. The most common rationale for including such a term as a divisor of the primary wavelength is to correct for fluctuations in the effective optical path length, generally caused by light scattering due to particulate matter in the sample. The second new term, at 1724 nm, corresponds to a weak protein absorption. This term, particularly in those samples with unusually high protein levels, may serve to correct for the contribution of protein absorptions to the intensity at 2152 nm.

For analytes that do not yield prominent absorptions in the spectra of the target specimens, the simple single-term Beer's law relationship fails completely. One solution in this case is again to assume a solution of the type represented by Equation (3), whereupon the task becomes to determine how many and which wavelengths/frequencies should be included in the analysis. One approach is to regress the set of spectral intensities, for each wavelength, against the analyte concentrations for the calibration specimens. The single wavelength that provides the best correlation with concentration is then taken as the "primary" wavelength, and further regressions are carried out holding the primary wavelength fixed to determine additional terms to complement the single-term model. The same process may be used to determine divisor terms (see the first term of Equation 4).

The stepwise regression approach to determining MLR terms is not guaranteed to find the optimal set of wavelengths, particularly for complex specimens where many terms may be required. The general problem is illustrated by the fact that in a set of calibration spectra, each comprising 2000 absorbance values, there are 2.5×10^{26} possible eight-term wavelength combinations. Brute-force evaluation of all possible eight-term MLR models is clearly out of the question, and there is an

ongoing search for more efficient methods.⁽⁷⁾ Recent developments include genetic algorithms to identify the optimal spectral regions. For example, an algorithm originally developed to identify diagnostic patterns in magnetic resonance spectra⁽⁸⁾ has been modified recently to seek out optimal spectral subregions for MLR.

3.2 Principal Component Regression and Partial Least Squares

The feature common to both of these approaches is that each spectrum is reduced to a sum of pseudospectra, or “loading vectors”. Each spectrum is newly represented by a unique set of “scores” – the set of coefficients required to reconstruct the original spectrum from the set of loading vectors. Typically, each of the spectra can be reconstructed to within the noise limits by a combination of typically 5–15 loading vectors, as compared to the hundreds or thousands of intensity values in the original spectra. The scores then provide the basis for quantitation.

The essential relationship in both the PCR and PLS models takes the form of Equation (5):

$$\mathbf{A} = \mathbf{T}\mathbf{B} + \mathbf{E}_A \quad (5)$$

With m spectra in the calibration set, each having n absorbance values, $\mathbf{A}$ is the $m \times n$ matrix of the calibration spectra. The spectra are reconstructed as a product of $\mathbf{B}$ ($h \times n$), the new basis set of loading vectors, and $\mathbf{T}$ ($m \times h$), the scores. To reiterate, the key to the process is that each spectrum is reduced from a vector of length n (a row in $\mathbf{A}$) to a new vector of length h (the corresponding row in $\mathbf{T}$), where h is typically between 5 and 15.

The column matrix of concentrations $\mathbf{c}$ is also related to the loading vectors $\mathbf{T}$, according to Equation (6):

$$\mathbf{c} = \mathbf{T}\mathbf{v} + \mathbf{e}_c \quad (6)$$

Here, $\mathbf{v}$ is the matrix of coefficients that relates the scores to the concentrations.

The reader is referred to several works in the literature^(9–14) for fuller explanations of PLS and PCR methods. For the sake of the present discussion, we note the following features common to the two methods:

- The main challenge in developing a method is to decide how many (and, in the case of PCR, which) loading vectors to include.
- The overall performance of either method may be improved by eliminating superfluous spectral regions from $\mathbf{A}$.
- The modeling of the spectra provides a means to detect outliers (those spectra with extraordinarily large spectral residuals $\mathbf{E}_A$).

3.3 Spectral Preprocessing

It is almost always necessary, or at least desirable, to preprocess the absorption spectra in some fashion; the aim is to enhance the spectral features that carry information regarding the analyte of interest, and effectively to suppress or eliminate superfluous features. The simplest form of “preprocessing” is the selection of appropriate wavelengths in MLR model development; the analogy in PLS and PCR is the selection of a limited spectral region (or regions).

The most common procedures are mean centering, variance scaling, and derivation. Mean centering simply subtracts the average of the calibration spectra from each of the individual spectra. Variance scaling involves first evaluating the standard deviation among spectra for the intensity at each wavelength. All spectra are then divided by the pseudospectrum of standard deviations, and hence scaled so that the variance is unity at all wavelengths. This operation effectively enhances the prominence of features due to species of relatively low concentration, while suppressing the intensities of strong (and variable) absorptions. The procedure is therefore most appropriate for the analysis of minor components. Derivation is commonly used to eliminate random fluctuations in the baseline (first derivative) and slope (second derivative) of the absorption spectra. Another benefit is the effective narrowing of spectral features, which may enhance specificity in the analytical method. Note that the features in the second-derivative spectrum are inverted relative to the absorption spectra. Although the second-derivative spectra plotted in Figure 5 have been inverted to yield peaks rather than valleys at positions corresponding to absorption peaks, this convention is not followed universally.

4 SERUM ANALYSIS

These analyses play a critical role in diagnosing and monitoring a wide variety of disorders (see Table 3), and a typical central hospital laboratory typically carries out many thousands of such tests every month. In order for a new testing procedure to be accepted clinically it must meet well-defined accuracy and precision standards. Although practical considerations such as the degree of automation also play a role in the acceptability of novel methods, these issues lie outside the scope of this article. In this section we present the current state of the art in the IR-based analysis of serum.

4.1 Infrared Spectroscopy of Serum

Among the most common clinical serum tests are those for the most abundant organic species. For at least

Table 3 Selected serum analytes that may be determined using IR spectroscopy

Analyte	Reference intervals ^a		Associated conditions ^a
Total protein	60–83 g L ⁻¹ (adult)	↑ ↓	Hypovolemic states Nutritional deficiency Liver disease Renal disease Fever Inflammation
Albumin ^b	32–48 g L ⁻¹ (adult)	↑ ↓	Dehydration Pregnancy
Urea	7–18 mg dL ⁻¹ (adult) (2.5–6.4 mmol L ⁻¹)	↑ ↓	Impaired kidney function Congestive heart failure Stress Severe liver damage Low protein diet Nephrotic syndrome
Glucose	65–105 mg dL ⁻¹ (3.6–5.8 mmol L ⁻¹)	↑ ↓	Diabetes mellitus Acute pancreatitis Stress/shock Pancreatic disorders Hepatic disease Extrapancreatic tumors
Cholesterol	150–235 mg dL ⁻¹ (male) ^c (3.9–6.1 mmol L ⁻¹)	↑	Idiopathic hypercholesterolemia Biliary obstruction Pregnancy
	141–219 mg dL ⁻¹ (female) ^c (3.6–5.7 mmol L ⁻¹)	↓	Hypothyroidism Severe liver damage Malnutrition Hyperthyroidism
Triglycerides	48–189 mg dL ⁻¹ (male) ^d (0.5–2.1 mmol L ⁻¹)	↑	Liver diseases Familial hyperlipidemia Alcoholism
	40–117 mg dL ⁻¹ (female) ^d (0.45–1.3 mmol L ⁻¹)	↓	Gout Malnutrition

^a From Wallach⁽¹⁵⁾ and Tietz.⁽¹⁶⁾ ↑ indicates conditions associated with levels above the reference range; ↓ indicates conditions associated with levels below the reference range.

^b Serum albumin levels generally parallel to total protein levels.

^c Desirable range (5th percentile to 75th percentile) for 40-year-old individuals. For men, the upper limit of the desirable range rises by approximately 1 mg dL⁻¹ for every year after 40; for women, the increase is ~3 mg dL⁻¹ for every year after 40.

^d Desirable range (reference interval is somewhat wider).

six of these, the MIR spectra are distinctive enough and the concentrations are high enough that they may be determined from the MIR spectra of serum. These include glucose, total protein, albumin, triglycerides, urea, and cholesterol. The basis for detecting and discriminating among the six analytes is illustrated by the spectra of the pure compounds shown in Figure 6. The NIR spectra also permit quantitation of the same six species.

Four comprehensive feasibility studies have been published, all of which differ in significant ways. Two were based upon MIR spectroscopy, and two on NIR spectroscopy. One MIR investigation used ATR spectroscopy, and another used dried serum films; the two NIR studies differed in more subtle, yet substantial, details.

4.2 Serum Analysis using Near-infrared Spectroscopy

Two major systematic investigations have been carried out. The first of these, reported in a pair of publications by Hall and Pollard,^(17,18) was based upon a rapid-scanning NIR spectrometer. The authors reported analytical methods for urea, triglycerides, total protein, and albumin.

Because the absorptions of protein overwhelm those of other dissolved species, it proved possible to use a simple MLR model to quantitate serum total protein. The absorption spectra of albumin further proved to be clearly distinguishable from those of the remaining proteins (primarily globulins; see Figure 7), so that a second two-term MLR model was sufficient to determine albumin levels. Based upon the second derivatives of the absorption spectra, the two models were as shown in

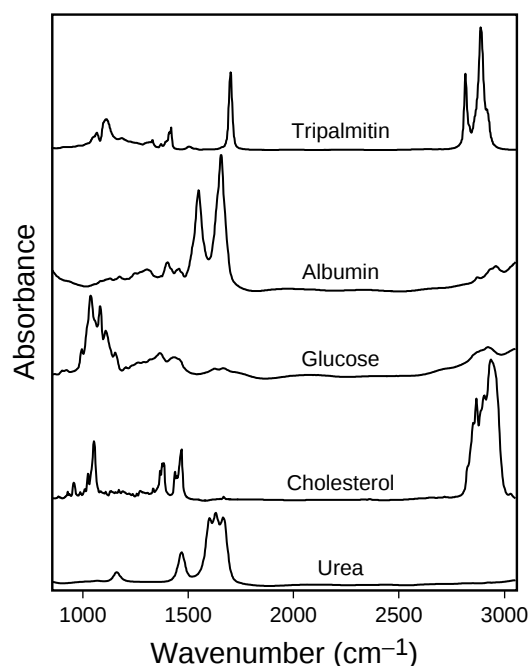


Figure 6 MIR absorption spectra for selected serum constituents. The spectra for urea, glucose, and albumin were acquired for aqueous solutions using an optical path length of 6 μm (the spectrum of water has been subtracted). Those for cholesterol and tripalmitin (tripalmitidoylglycerol) were measured for solutions in carbon tetrachloride using an optical path length of 0.5 mm.

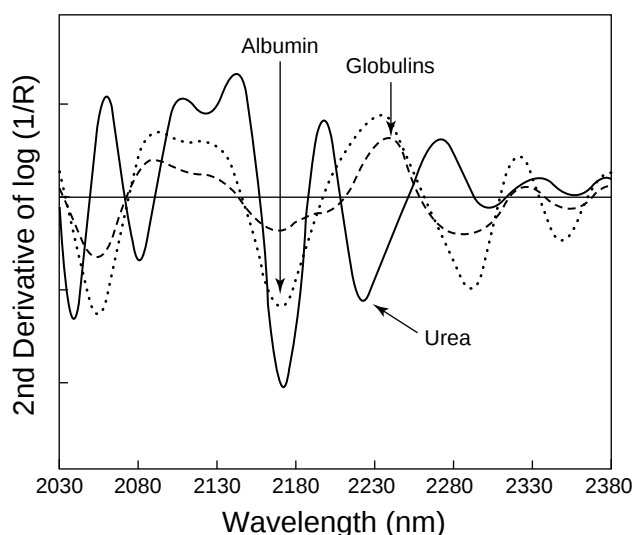


Figure 7 NIR reflectance spectra (second derivatives) for albumin, globulins, and urea. Total serum protein may be quantified by the intensity of the serum absorption at 2064 nm, corresponding to minima (absorption maxima) in the albumin and globulin second-derivative spectra. (Adapted by permission of Elsevier Science from J.W. Hall, A. Pollard, 'Near-infrared Spectroscopic Determination of Serum Total Proteins, Albumin, Globulins, and Urea', *Clinical Biochemistry*, 483–490, Vol. 26, © 1993 by the Canadian Society of Clinical Chemists.)

Equations (7) and (8):

$$C_{\text{albumin}} = 15 - 4419A(2178 \text{ nm}) + 3655A(2206 \text{ nm}) \quad (7)$$

$$C_{\text{total protein}} = 65 - 7821A(2064 \text{ nm}) - 2373A(1440 \text{ nm}) \quad (8)$$

The protein levels predicted by the NIR model are compared to the reference analytical results in Figure 8.

The models for urea and triglyceride quantitation, also based upon the second-derivative spectra, required eight and eleven PLS factors, respectively. The spectral regions employed as the basis for these models differed slightly: optimal for urea quantitation (Figure 8) was a combination of the ranges 1324–1800 and 2304–2370 nm, whereas triglyceride levels were optimally predicted by combining the ranges 1635–1800

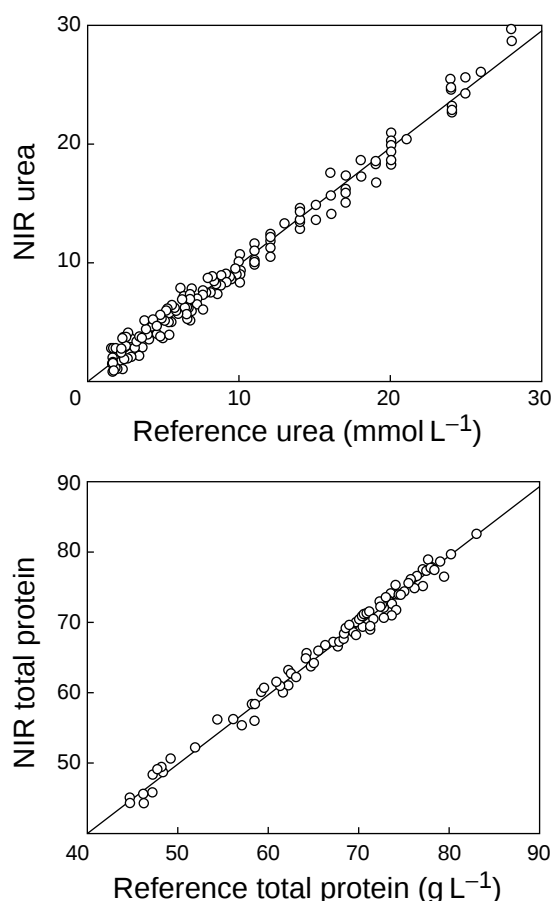


Figure 8 NIR-predicted serum urea and serum protein levels compared to reference analytical results (see also "NIR A" in Table 4). The line of identity is included. (Adapted by permission of Elsevier Science from J.W. Hall, A. Pollard, 'Near-infrared Spectroscopic Determination of Serum Total Proteins, Albumin, Globulins, and Urea', *Clinical Biochemistry*, 483–490, Vol. 26, © 1993 by the Canadian Society of Clinical Chemists.)

and 2035–2375 nm. The 1850–2025 nm range, spanning a very intense water absorption, was explicitly excluded from both models. This provides an example of how PLS modeling benefits from the exclusion of spectral segments that carry no relevant analytical information. These NIR analytical methods for serum total protein, albumin, urea, and triglycerides are summarized as part of the synopsis of methods presented in Table 4.

A more recent NIR investigation was identical in spirit to the inaugural studies but incorporated one substantial change in the experimental protocol.⁽¹⁹⁾ Although the early work was carried out using an optical path length of 0.5 mm, the more recent work used a path length of 2.5 mm. It is quite counter-intuitive to expect improved accuracy at this relatively long path length, because the region richest in solute absorptions (2050–2450 nm) is bordered by two strong water absorptions (see Figure 2). The accessible range within this window is substantially reduced as the optical path length is increased, by virtue of the further encroachment of the shoulders of the two water absorptions. It emerged, however, that this effect was more than compensated for by the enhanced signal-to-noise for solute absorptions in the spectral window that remained accessible.

Figure 9 demonstrates the accuracy of the second NIR study in assays for triglycerides, urea, and cholesterol. Although the analytical methods for total protein and albumin also proved successful, the attempt to quantitate serum lactate proved to be fruitless. The poor results for lactate are largely due to the relatively low serum concentration. Another contributing factor may be that the NIR spectrum is not rich enough to differentiate lactate from other dissolved species (the only NIR bands arise from the methyl group). The analytical methods for the other six analytes are summarized in Table 4. As indicated in Table 4, all methods were based upon PLS models and all made use of the same 2062–2353 nm spectral region.

The NIR quantitation of glucose is of extraordinary interest. This interest stems from the early promise of NIR spectroscopy as a means of monitoring blood glucose levels noninvasively. Indeed, one of the primary aims of the *in vitro* study⁽¹⁹⁾ was to delineate better the ability of NIR spectroscopy to quantitate serum glucose under ideal experimental conditions. The success of this endeavor is summarized in Figure 10, which superimposes a Clarke error grid⁽²⁰⁾ on the scatterplot comparing NIR to reference glucose levels. The error grid serves as a template indicating regions corresponding to acceptable

Table 4 Serum analyses using MIR and NIR spectroscopy

Analyte	Method ^a	PLS spectral region(s)	No. of PLS factors	SEP (mmol L ⁻¹) ^b
Glucose	ATR	950–1200 cm ⁻¹	9	0.58
	Film MIR	925–1250 cm ⁻¹	10	0.41
	NIR B	2062–2353 nm	13	1.3
Triglycerides	ATR	1100–1500, 1700–1800 cm ⁻¹	13	0.11
	Film MIR	900–1500, 1700–1800, 2800–3000 cm ⁻¹	7	0.23
	NIR A	1635–1800, 2035–2375 nm	8	0.19
	NIR B	2062–2353 nm	13	0.11
Total protein	ATR	1350–1700 cm ⁻¹	3	1.4
	Film MIR	900–1800 cm ⁻¹	13	2.8
	NIR A	2064, 1440 nm (MLR model)	(2) ^c	1.7
	NIR B	2062–2353 nm	10	2.3
Albumin	Film MIR	1100–1800 cm ⁻¹	12	2.2
	NIR A	2178, 2206 nm (MLR model)	(2) ^c	1.1
	NIR B	2062–2353 nm	7	2.0
Cholesterol	ATR	2800–3000 cm ⁻¹	8	0.22
	Film MIR	1100–1300, 1700–1800, 2800–3000 cm ⁻¹	11	0.28
	NIR B	2062–2353 nm	13	0.32
Urea	ATR	1130–1800 cm ⁻¹	20	0.48
	Film MIR	1400–1800 cm ⁻¹	13	1.1
	NIR A	1324–1800, 2304–2370 nm	11	0.8
	NIR B	2062–2353 nm	12	0.46

^a “ATR” = MIR ATR spectroscopy of native serum;⁽²¹⁾ “Film MIR” = MIR spectroscopy of dried serum films;⁽²³⁾ “NIR A” = NIR spectroscopy of native serum at 0.5-mm path length;^(17,18) “NIR B” = NIR spectroscopy of native serum at 2.5-mm path length.⁽¹⁹⁾

^b Standard error of prediction (SEP) for independent test sets except for “ATR” study, where the standard error of cross-validation for the calibration set is given.

^c Two wavelength terms were required for the albumin and total protein MLR calibration models.

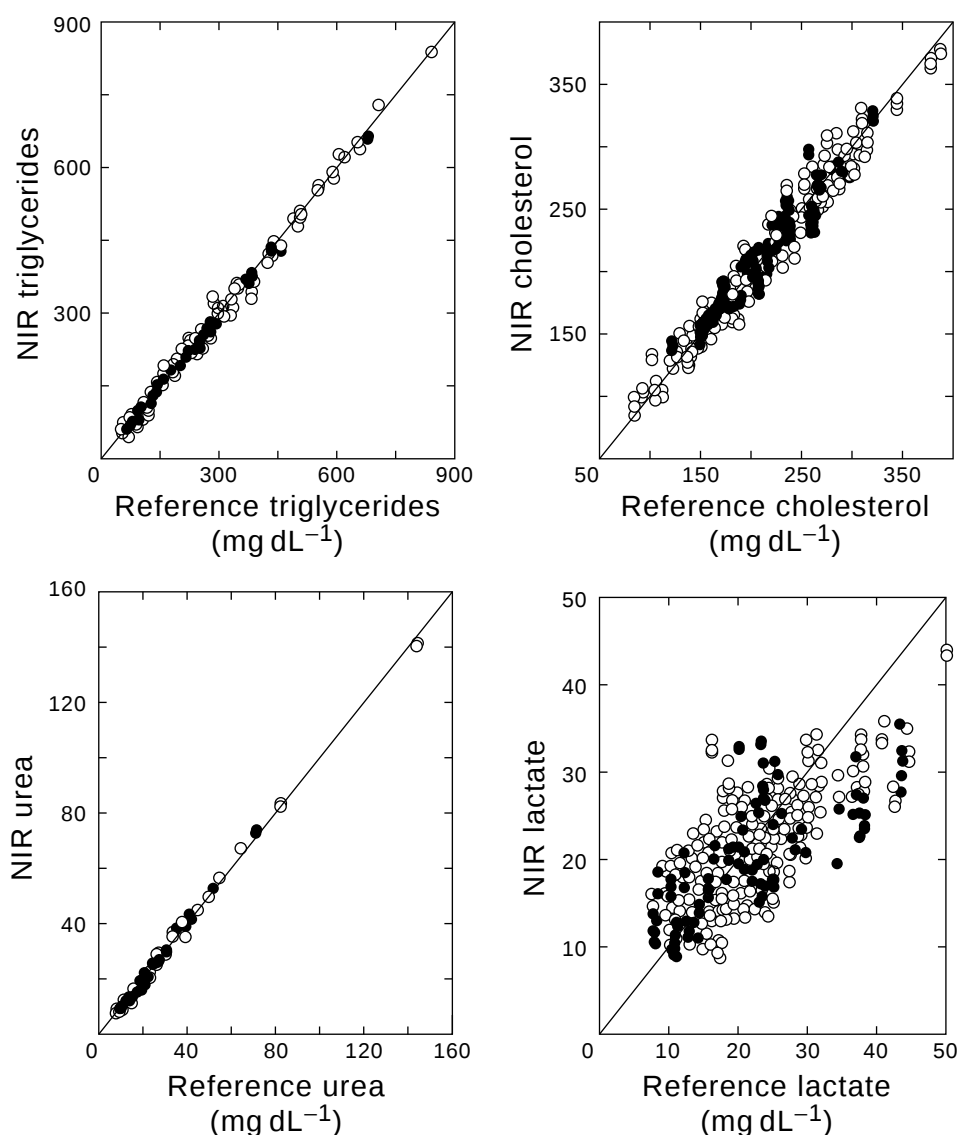


Figure 9 Comparison of NIR-predicted serum analyte levels to reference analytical results (see also “NIR B” in Table 4). Open circles correspond to the calibration (training) set, solid circles to the validation (test) set and the solid line is the line of identity. (Adapted from K.H. Hazen, M.A. Arnold, G.W. Small, ‘Measurement of Glucose and Other Analytes in Undiluted Human Serum with Near-infrared Transmission Spectroscopy’, *Analytica Chimica Acta*, 255–267, Vol. 371, © 1998, with permission from Elsevier Science.)

analytical errors (A, B) and regions corresponding to errors that would lead to dangerous or fatal clinical decisions (C, D, E). As the authors point out, this analytical method is not accurate enough to meet clinical demands but it is accurate enough to suggest that further investigation is warranted.

4.3 Serum Analysis using Mid-infrared Spectroscopy

The two comprehensive feasibility studies have followed two different paths to avoid the problems associated with transmission spectroscopy of the native serum. One of

these made use of ATR spectroscopy of the liquid, and the second was based upon transmittance spectroscopy of dried serum films.

4.3.1 Attenuated Total Reflectance Spectroscopy of Native Serum

In this work the investigators sought to quantify glucose, total protein, cholesterol, triglycerides, urea, and uric acid on the basis of the MIR ATR spectra collected using a CIRCLE ATR cell (Spectra-Tech Inc., Shelton, CT, USA).⁽²¹⁾ It had been concluded on the basis of

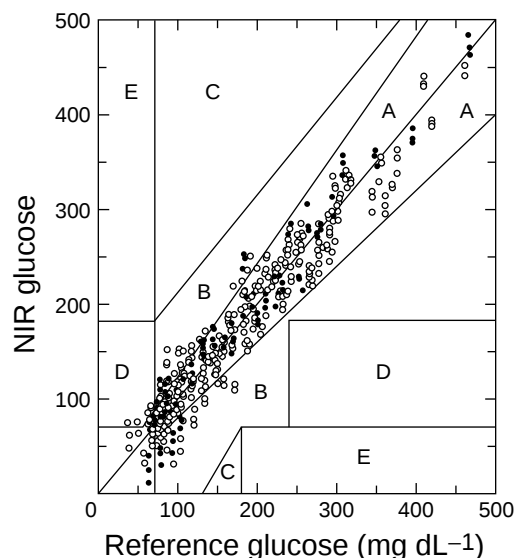


Figure 10 NIR-predicted serum glucose levels vs reference assays (see also “NIR B” in Table 4). Open circles correspond to the calibration (training) set, solid circles to the validation (test) set, and the solid line is the line of identity. A Clarke error grid⁽³⁰⁾ is superimposed, distinguishing regions corresponding to clinically safe analytical errors (regions A, B) from analytical errors that would result in dangerously inappropriate clinical decisions (C, D, E). (Adapted from K.H. Hazen, M.A. Arnold, G.W. Small, ‘Measurement of Glucose and Other Analytes in Undiluted Human Serum with Near-infrared Transmission Spectroscopy’, *Analytica Chimica Acta*, 255–267, Vol. 371, © 1998, with permission from Elsevier Science.)

an earlier study⁽²²⁾ that the most critical factor in this procedure is careful cleaning of the zinc selenide ATR optical element between spectral acquisitions. Therefore, the more recent investigation included a cleaning cycle using first a detergent solution, then distilled water, and finally ethanol. The element was then dried by pressurized nitrogen before admitting the next sample. Finally, the specimen was allowed 30 s to reach thermal equilibrium (the cell temperature was kept at $37 \pm 0.02^\circ\text{C}$) before the spectrum was acquired.

The accuracy of this approach is illustrated by the scatterplots in Figure 11. All of the IR-predicted concentrations were based upon PLS models, summarized as part of Table 4. The MIR ATR approach is substantially more accurate than NIR for the quantitation of serum glucose. Why is this the case? The answer relates to the nature of the glucose structure. A rich set of strong absorptions appears in the MIR region of $950\text{--}1250\text{ cm}^{-1}$, corresponding to skeletal C–O stretching vibrations, whereas the NIR spectrum shows only a single absorption in the CH combination region (see Table 1) centered at 2270 nm . The combination bands involving glucose OH groups are very diffuse and are overlapped with the water absorption

at 1935 nm to such an extent that they are essentially valueless.

4.3.2 Transmittance Spectroscopy of Dried Serum Films

When a small volume of serum is spread evenly on an IR-transparent window and allowed to dry, the resulting film may be used as the basis to quantitate at least six analytes. A large study based upon this approach used 200 specimens as the basis to develop PLS calibration models for glucose, triglycerides, total protein, albumin, cholesterol, and urea, and an additional 100 specimens to test the accuracy of these models.⁽²³⁾

A representative spectrum is shown in Figure 4. This spectrum is for a film dried from a 50 : 50 mixture of serum and aqueous potassium thiocyanate solution (4 g L^{-1}); the prominent absorption at 2060 cm^{-1} originates with the SCN^- ion. All serum specimens were diluted in this fashion prior to measurement, with the objective of using the absorption intensity at 2060 cm^{-1} to normalize the spectra and hence compensate for possible imprecision in preparation of the films. The specimens were prepared for IR spectroscopy by spreading $7\text{ }\mu\text{L}$ of the diluted serum evenly on the surface of a 13-mm -diameter BaF_2 window. Duplicate samples were prepared in each case, and the corresponding spectra were averaged for PLS analysis.

The PLS trials were preceded by a normalization stage (all spectra were normalized to a common integrated intensity in the SCN^- absorption at 2060 cm^{-1}). The second derivatives of the normalized spectra then served as the basis for the analyses. Scatterplots comparing the reference analytical levels to the IR-predicted albumin, total protein, glucose, cholesterol, triglycerides, and urea are shown in Figure 12. The corresponding PLS models and their analytical accuracies are compiled in Table 4. Attempts to quantitate uric acid and creatinine proved unsuccessful due to their relatively low serum concentrations.

We use the example of glucose to illustrate the procedure that is used to gauge the appropriate number of PLS factors to include in the final model. Recalling that the pool of spectra is divided into a calibration set of 200 samples and a validation set of 100 samples, the aim was to arrive at a final model that optimally extracted the analytical information latent in the calibration spectra. To guard against the possibility of overfitting, the IR-predicted analytical levels were typically compared to reference values for models with 1–15 factors. The trends illustrated in Figure 13 are typical; the standard error of calibration (SEC) in the calibration set specimens decreases rapidly as the initial factors are added, and then tends to plateau as all of the analytically relevant factors were extracted. Additional factors provide rapidly

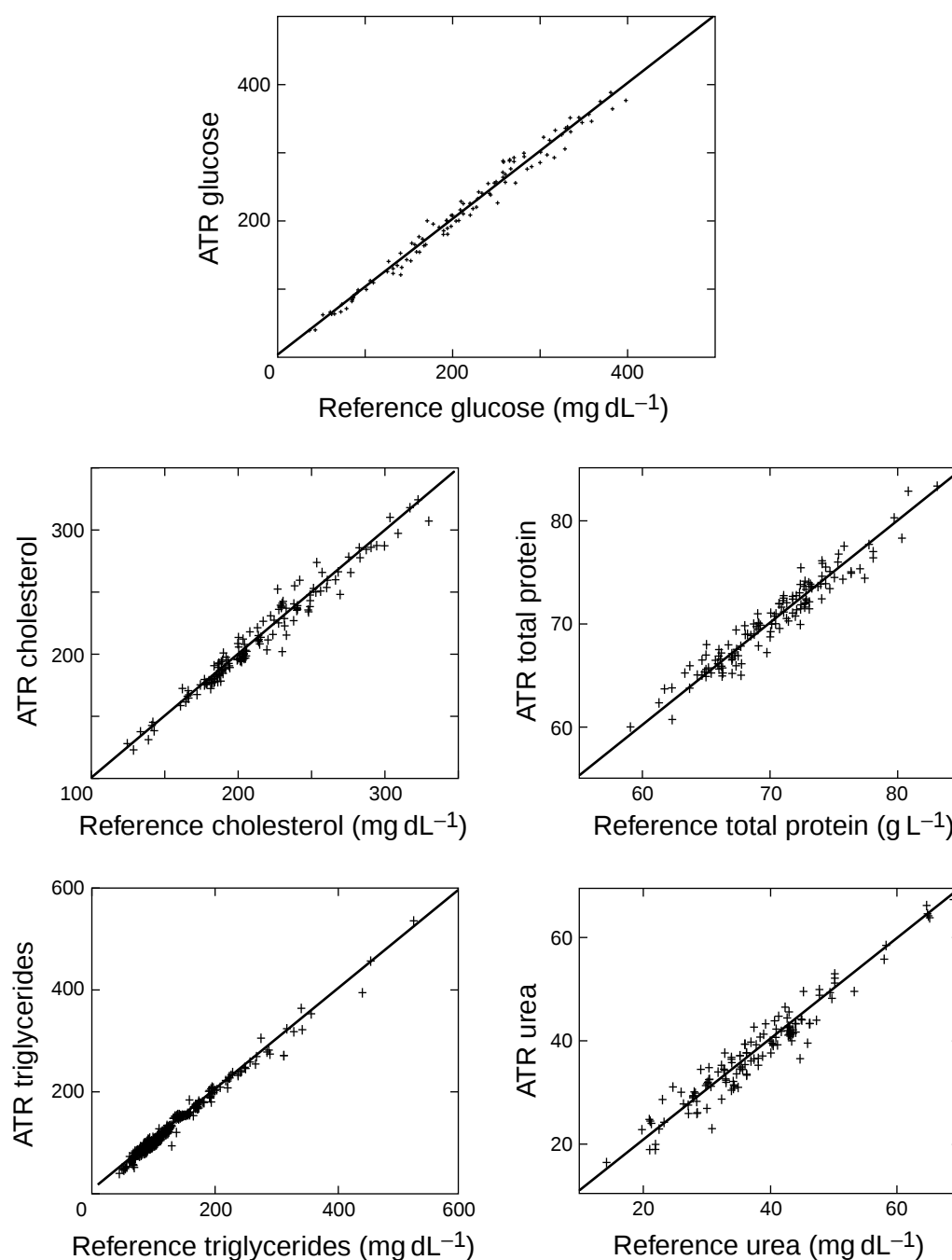


Figure 11 Serum ATR MIR-predicted glucose, total protein, cholesterol, urea, and triglycerides plotted against the gold standard clinical assays, with the line of identity included for reference (see also “ATR” in Table 4). (Adapted from Heise et al.⁽²¹⁾ by permission of the Society for Applied Spectroscopy.)

diminishing returns, and generally model spectral features that are unique to the set of calibration spectra (i.e. noise). This may be inferred by examining the corresponding trend in the standard error of prediction (SEP) for the validation set. Although the SEC and SEP are essentially identical for all models up to and including 10 factors, this is no longer the case for models including 11 factors

or more – although the errors continue to diminish for the calibration set, the opposite trend takes hold for the validation set. The appropriate number of PLS factors corresponds to the point at which the standard error in the validation set begins to increase; this model, corresponding to 10 PLS factors, is equally accurate for the samples in the calibration and validation sets

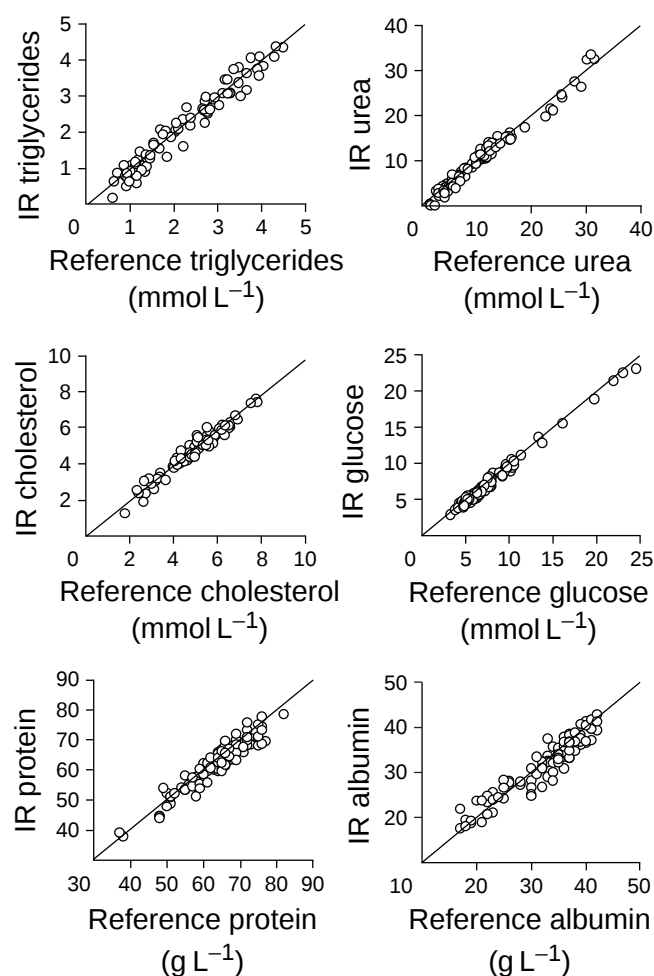


Figure 12 Scatterplots comparing MIR-predicted serum analyte levels to reference analytical results (see also “Film MIR” in Table 4). The line of identity is also plotted. The spectra were acquired for dried serum films (see Shaw et al.⁽²³⁾).

(Figure 14). The serum PLS models determined in this fashion for the dried serum films varied from a 7-factor model for triglycerides to 13 factors for urea and total protein (see Table 4).

Finally, the example of serum glucose provides a good demonstration of a useful feature of PLS modeling. The first PLS weight vector is a least-squares estimate of the spectrum of the analyte of interest; it is a weighted sum of all the calibration spectra, with the weights being the reference concentrations.⁽¹²⁾ If this estimate shows similarity to the spectrum of the pure compound, it may be inferred that the PLS model is soundly based in that it is incorporating spectroscopic patterns that originate with that species. To illustrate this, we have evaluated a PLS model for glucose based upon the absorption spectra (not their derivatives) of the dried films. The first PLS weight vector is plotted in Figure 15 together with the absorption spectrum for an aqueous glucose solution. The striking

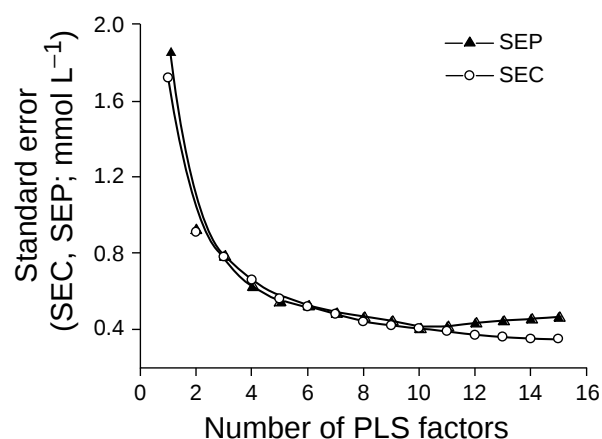


Figure 13 Trends in the SEC and SEP with increasing number of PLS factors for a glucose quantitation model. The 10-factor model was chosen as optimal, based upon these trends (see Shaw et al.⁽²³⁾).

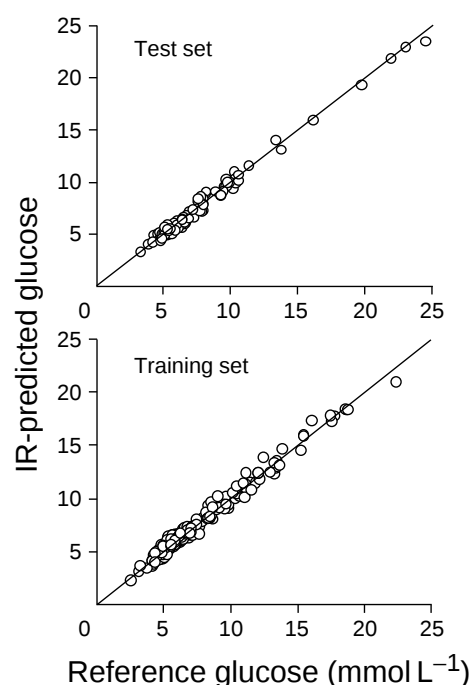


Figure 14 Comparison of the scatterplots comparing MIR-predicted serum glucose levels to reference analytical results for the training (calibration) and test (validation) sets. Results are based upon the spectra of dried serum films using the 10-factor PLS model (see Figure 13 and “Film MIR” in Table 4), and the line of identity is superimposed on each plot.

similarities between these two traces provide a good illustration of how this PLS weight vector can be used to support the validity of the PLS model as a whole. At the same time, it should be emphasized that the first weight vector does not always show such a strong resemblance to the IR spectrum of the target analyte. Particularly

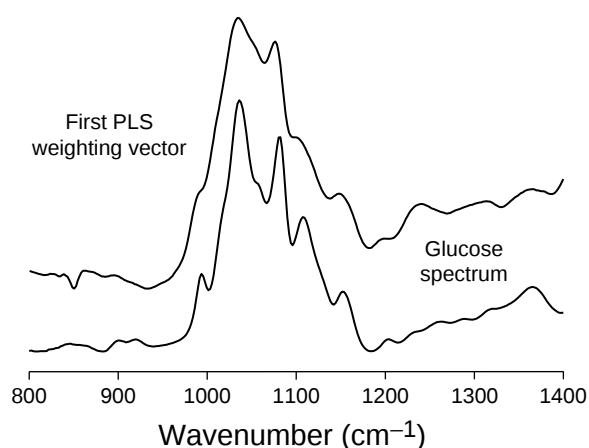


Figure 15 Comparison of the spectrum of an aqueous glucose solution to the first weighting vector for a serum glucose PLS calibration model. Comparisons of this type can provide confirmation, as in this case, that PLS modeling is soundly based upon genuine spectroscopic features of the analyte of interest rather than chance correlations.

for weakly absorbing species of low concentration, the first weight vector—even for a soundly based PLS model—may incorporate compensating features from other components of the mixture that are as large as or larger than features ascribed to the species of interest.

5 SERUM AND BLOOD GLUCOSE

The analysis for glucose is probably the most common blood/serum test. Much of the demand arises from the requirement for frequent self-testing in the diabetic population, and the majority of attempts to quantify glucose using IR spectroscopy have been motivated by the prospect of a noninvasive *in vivo* test. Because NIR radiation penetrates tissue to depths of millimeters or more, and because an absorption spectrum may be measured for living tissue by using fiber optics or other arrangements, hope has been held out for a NIR method to quantitate blood glucose *in vivo*.^(24,25) Although this subject matter is reviewed elsewhere in this encyclopedia, the various *in vitro* NIR laboratory studies provide some interesting insights regarding the prospects for *in vivo* measurement.

In principle, serum or blood glucose may be quantified either by using MIR spectroscopy or by exploiting any of three sets of NIR absorptions, namely those corresponding to vibrational combination bands (2000–2500 nm), the first overtone absorptions (1400–1800 nm), or the second overtone bands (950–1250 nm). All of these have been explored in attempting to quantitate serum glucose,⁽²⁴⁾

Table 5 IR spectroscopic determinations of glucose in whole blood

Study	Optical path length	Spectral range	No. of PLS factors	SEP (mM)
MIR 1 ⁽²⁶⁾	ATR	750–1500 cm ⁻¹	16	1.1
MIR 2 ⁽²⁷⁾	ATR	750–1500 cm ⁻¹	ANN ^a	0.9
MIR 3 ⁽²⁸⁾	ATR	950–1200 cm ⁻¹	11	0.8
NIR ⁽²⁹⁾	1 mm	1515–1818, 2062–2353 nm	8	2.1

^a Artificial neural network model.

but there are surprisingly few published studies attempting to quantitate glucose in whole blood. Three MIR ATR investigations^(26–28) yielded methods with standard errors of 0.8–1.1 mM, whereas the lone report addressing the use of NIR spectroscopy for the measurement of glucose in whole blood reported a SEP of 2.2 mM.⁽²⁹⁾ The PLS models are summarized in Table 5.

How accurate must the glucose analysis be in order to be acceptable clinically? One set of guidelines for clinical testing that has been adopted widely is that of Barnett.⁽³⁰⁾ Under those guidelines, a new analytical method for blood or serum glucose method should agree with established methods with a maximum standard deviation of 0.28 mM. A more detailed examination of the clinical consequences of inaccurate glucose testing in diabetics has provided the “Clarke grid”⁽²⁰⁾ (see Figure 10). Although the scatterplot superimposed on this grid represents a serum NIR analytical method with a standard deviation (SEP) of 1.3 mM relative to reference analyses (substantially larger than the allowable error limit suggested by Barnett), the method clearly approaches the criteria for acceptability set out by Clarke et al. As mentioned earlier in this article, the regions labelled A and B in Figure 10 correspond to clinically acceptable errors. The most serious deficiency of the NIR method is in the analyses for specimens with lower glucose concentrations.

To summarize the present state of the art: the most accurate NIR analysis of serum glucose, carried out using the spectral window and transmission path length optimal for NIR detection, approaches the level of accuracy required for clinical use; and the NIR detection of glucose in blood is less accurate, mainly due to the confounding influence of light scattering by the blood cells.

6 FETAL LUNG MATURITY DETERMINED BY INFRARED SPECTROSCOPY

Among the most common concerns with problematic pregnancies is the possibility that the baby, if born

prematurely, will suffer from respiratory distress syndrome. Failing to produce pulmonary surfactant properly, these infants have traditionally been at high risk of severe respiratory problems or even death. It was recognized in the late 1970s that the fetal lung maturity could be estimated by analyzing for lung surfactants in the amniotic fluid. These tests have been used as the basis for the clinical decision as to whether and when to induce labor, balancing the risk to the mother in continuing the pregnancy with the benefit to the fetus of further lung development within the womb.

The test that has gained widest acceptance is the determination of the amniotic fluid lecithin/sphingomyelin ratio, using thin-layer chromatography (TLC). By its nature, this is a time-consuming and labor-intensive test, and an alternative based upon fluorescence depolarization has been proposed and widely adopted. This procedure measures the ratio of surfactant to protein in amniotic fluid.

Both the lipid and protein constituents provide clear absorptions in the MIR spectra (Figure 16), and both the lecithin/sphingomyelin ratio⁽³¹⁾ and the surfactant/protein ratio⁽³²⁾ may be determined from the IR spectra of dry amniotic fluid films. For the lecithin/sphingomyelin ratio determination, the values predicted from the IR spectra (a 14-factor PLS model incorporating the spectral region 2800–3200 cm⁻¹) showed a very good correlation ($r = 0.90$) with the TLC values. Similarly, when surfactant/protein ratios (determined using the Abbott TDx analyzer) were used to calibrate the PLS model, the resulting IR-based analytical method closely reproduced the reference TDx assays (Figure 17).

By their nature, both the TLC and TDx reference methods are inherently less precise than, for example, the common serum assays. For that reason, the scatterplots illustrated in Figure 17 inevitably reflect imprecision in the reference analyses. To confirm that the PLS model

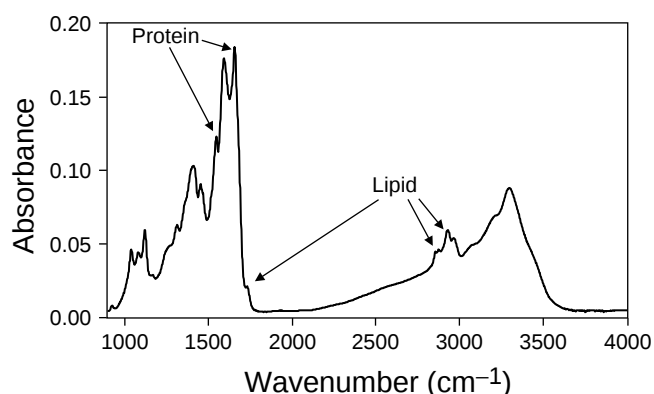


Figure 16 Representative MIR absorption spectrum of a dried amniotic fluid film.

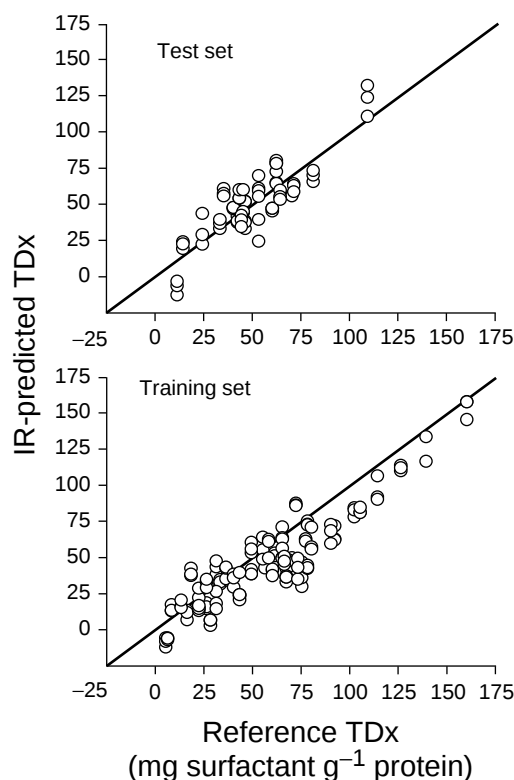


Figure 17 Scatterplots summarizing a PLS model for the determination of amniotic fluid surfactant/protein ratio from the MIR spectra of dried films.⁽³²⁾ The sloping line in each plot is the line of identity.

is based upon the spectral features of surfactant constituents, and is not built upon an accidental correlation with reference analyses, Figure 18 compares the first PLS weight vector to an experimental spectrum. The region plotted in Figure 18 corresponds to CH stretching vibrations of the amniotic fluid constituents, and therefore includes the characteristic vibrations of the long lipid methylene chains as well as absorptions from the proteins. The experimental trace is a difference spectrum, obtained by subtracting the average of all IR spectra corresponding to surfactant/protein ratios less than 55 mg g⁻¹ from the average of those with surfactant/protein ratios above that value. The similarity between these two traces confirms that the PLS model is founded upon genuine spectral features originating with the lipid and protein constituents of amniotic fluid.

7 OTHER FLUIDS

7.1 Urine Analysis

Two of the most common analytical tests are for urine creatinine and protein, both of which are key indicators

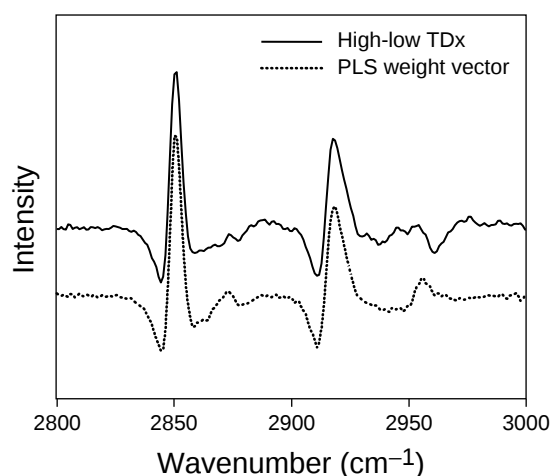


Figure 18 Validation of a PLS model to quantitate the amniotic fluid surfactant/protein ratio from MIR spectra of dried amniotic fluid films. The lower trace is the first PLS weight vector for a MIR quantitation model calibrated with reference to laboratory TDx measurements of the surfactant/protein ratio. The upper trace is a difference spectrum, obtained by subtracting the average of all IR spectra corresponding to surfactant/protein ratios of less than 55 mg g^{-1} from the average of those with surfactant/protein ratios above that value. The $2800\text{--}3000 \text{ cm}^{-1}$ region proved nearly optimal for PLS calibration, with only marginal improvements gained by the addition of segments in the $900\text{--}1800 \text{ cm}^{-1}$ region (see Figure 16).

Table 6 Selected urine analytes that may be determined using IR spectroscopy

Analyte	Reference intervals ^a
Urea	12–20 g 24 h^{-1} (428–714 mmol 24 h^{-1})
Creatinine	1–1.6 g 24 h^{-1} (8.8–14.2 mmol 24 h^{-1})
Total protein	<0.1 g 24 h^{-1}

^a From Wallach⁽¹⁵⁾ and Tietz.⁽¹⁶⁾ Diagnostic information is generally inferred from totals passed in urine over a 24-h period. Concentrations for the NIR study reported in Shaw et al.⁽⁶⁾ were grouped in the ranges $100\text{--}400 \text{ mmol L}^{-1}$ (urea), $2.5\text{--}12.5 \text{ mmol L}^{-1}$ (creatinine) and $0\text{--}3 \text{ g L}^{-1}$ (protein).

of kidney function (Table 6). Urea is also measured for the same reason, although less frequently. Because the normal physiological concentration range is quite high for urea, intermediate for creatinine, and very low in the case of protein, each of these three analytes posed unique challenges in developing analytical methods based upon the NIR spectra of the native fluid.⁽⁶⁾

As discussed earlier, a simple MLR model sufficed to quantitate urea across the physiological range. A 9-factor PLS model was required to determine creatinine with a

SEP of 0.79 mmol L^{-1} . The best that could be achieved for protein was an 8-factor PLS model with a SEP of 0.23 g L^{-1} . This model is clearly insufficient to provide the information that is sought most often clinically, because a protein level above $\sim 0.1 \text{ g L}^{-1}$ is considered to be a warning sign of possible kidney malfunction. In an effort to improve the accuracy of the IR-based method for samples with low protein concentrations, a second PLS model was derived using only those specimens with reference protein concentrations of less than 1 g L^{-1} . Although the result was an improved accuracy in quantitating protein in the range $0\text{--}1 \text{ g L}^{-1}$, the SEP of 0.12 g L^{-1} remained unacceptably high. With a detection limit of 0.36 g L^{-1} (taken as three times the standard error), many urine specimens have protein levels below the threshold that is detectable by NIR spectroscopy.

7.2 Saliva

Because it is so readily available, saliva has often been considered as a potential source of diagnostic information.⁽³³⁾ The MIR spectrum of the dried film (Figure 19) reveals not only the protein constituents but also thiocyanate (SCN^-). Although it is somewhat surprising to the layman to learn that this ion is present in appreciable amounts in human saliva, it plays a functional role; enzymatic conversion yields salivary hypothiocyanate (OSCN^-), which is a highly effective antibacterial agent.

The SCN^- ion shows an absorption in a spectral region that is typically devoid of any other bands. As a result, it proved possible to quantitate this ion in saliva through a simple Beer's law relationship according to

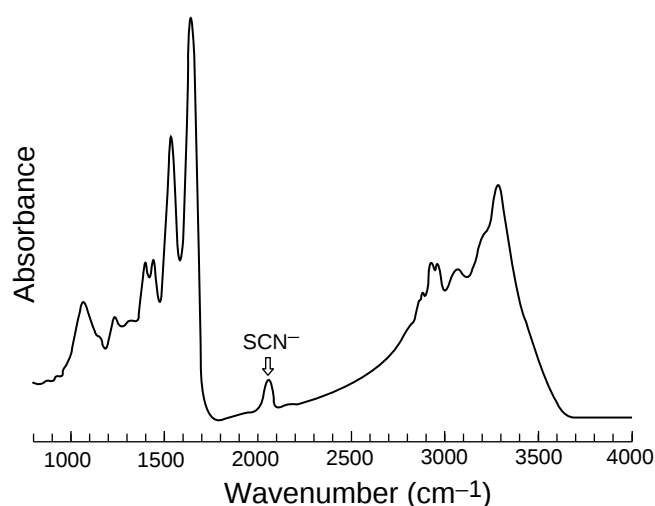


Figure 19 Representative MIR spectrum of a dried saliva film. The absorption at 2060 cm^{-1} is from endogenous thiocyanate (SCN^-).⁽³⁴⁾

Equation (9):⁽³⁴⁾

$$C_{\text{SCN}^-} = -0.001 + 0.65A(2058 \text{ cm}^{-1}) \quad (9)$$

where $A(2058 \text{ cm}^{-1})$ denotes the integrated intensity for absorption at 2058 cm^{-1} for a 5- μL saliva aliquot dried to a spot of diameter 4 mm. The same study indicated further that saliva thiocyanate concentration levels may follow a circadian rhythm, with maximum levels early in the day.

8 DISEASE DIAGNOSIS BASED ON INFRARED SPECTRAL PATTERN RECOGNITION

Although the analysis of biological fluids has a long tradition in providing information to suggest or corroborate diagnosis, a complementary technique is emerging for the interpretation of the IR spectra. Rather than deriving analyte levels explicitly from them, the spectra may be viewed as fingerprints that correlate directly with the presence or absence of disease. Because the spectra are complex, patterns characteristic of specific diseases are rarely (if ever) discernable from visual examination of the spectra. However, multivariate analytical methods may identify subtle patterns distinguishing the spectra corresponding to “normal” specimens from those corresponding to diseased patients.

The general procedure for developing this diagnostic test has much in common with the techniques employed to develop IR-based analytical methods. The first step is to acquire appropriate specimens from two sets of donors. One set of normal or control samples is required, whereas the second set corresponds to patients who have been diagnosed by traditional methods as having the disease of interest. The corresponding IR spectra are collected and subjected to two interlinked procedures: feature extraction and classification. The feature extraction procedure identifies characteristics that distinguish the normal spectra from diseased, whereas the aim of the classification stage is to separate optimally the two groups of spectra based upon those characteristics. Finally, the general applicability of the optimal classifier is tested by predicting the class assignments (normal or diseased) for a separate group of test spectra and comparing these a posteriori to the “gold standard” diagnoses.

8.1 Arthritis Diagnosis from Infrared Spectroscopy of Synovial Fluid

Synovial fluid is an ultrafiltrate of blood plasma that serves to transport nutrients to cartilage as well as to lubricate the joints. It is readily available for diagnostic testing (the

fluid is commonly drained from joints that are inflamed, either as a result of disease or physical trauma), however there is no synovial fluid analytical test or combination of tests to reliably diagnose arthritis or to distinguish arthritic conditions from one another.

Two studies have suggested that the IR spectra of synovial fluid specimens provide the basis to diagnose arthritis and to differentiate among its variants.^(35,36) A NIR study demonstrated that osteoarthritis, rheumatoid arthritis, and spondyloarthropathy could be distinguished on the basis of the synovial fluid absorption patterns in the range 2000–2400 nm.⁽³⁵⁾ In that case, the pool of synovial fluid spectra was subject to principal component analysis, and eight principal component scores for each spectrum were employed as the basis for linear discriminant analysis (LDA). On that basis, the optimal LDA classifier matched 105 of the 109 spectra to the correct clinical designation (see Table 7).

An investigation based upon the MIR spectra of dried synovial fluid films showed similar success in distinguishing spondyloarthropathy, rheumatoid arthritis, and osteoarthritis from one another and from control specimens (generally synovial fluid aspirates from individuals with injuries rather than diseased joints).⁽³⁶⁾ This study is of interest in that it made use of a genetic region selection algorithm⁽⁸⁾ to identify a set of 15 discrete spectral sub-regions differentiating the 4 classes of spectra. With each spectrum represented by a set of 15 regional intensities, LDA provided the basis for successful classification.

Although applications of this type are not analytical in the traditional sense, they may provide analytical information indirectly. The successful classification of IR spectra according to disease type implies that the composition of the specimen is altered in a characteristic fashion with the onset of disease – in this case the synovial fluid make-up reflects the presence and type of arthritis. This is an intriguing finding, particularly as it is very unlikely that the IR spectra are detecting any particular constituent that cannot be (and has not been) assayed

Table 7 Classification table: clinical versus IR-based arthritis diagnoses

		IR-based diagnoses ^a		
		SA	RA	OA
Clinical diagnoses	SA	12	3	0
	RA	0	65	0
	OA	0	2	27

^a The IR-based diagnoses are from principal component analysis and LDA of synovial fluid NIR spectra (see text): SA = spondyloarthropathy; RA = rheumatoid arthritis; OA = osteoarthritis. The table indicates, for example, that of the 29 spectra corresponding to patients with osteoarthritis, 27 were classified correctly but two were misclassified as rheumatoid arthritis. (see Shaw et al.⁽³⁵⁾).

previously. The strength of this approach therefore lies not in the ability of IR spectroscopy to identify novel disease markers, but rather in the ability of multivariate pattern recognition and classification methods to perceive characteristic changes in the balance among the IR-detectable components.

8.2 Disease Pattern Recognition in Mid-infrared Spectra of Serum

The premise underlying this research is that the combination of serum MIR spectroscopy with pattern recognition methods can distinguish healthy subjects from those with specific disease types. One report has demonstrated success rates of better than 90% in distinguishing diabetics from healthy subjects, type I from type II diabetics, and patients with rheumatoid arthritis from healthy subjects.⁽³⁷⁾ Again, this work is intriguing because it is very unlikely that the IR spectra reveal any fundamentally new serum constituents as “disease markers”. Although the successful classifications indicate that the presence of disease is linked to specific relationships among specific spectral features, the challenge remains to interpret the physiological significance of those features. As research progresses in this area, we may anticipate that these characteristic relationships among spectral features will be interpreted to reveal characteristic relationships among serum metabolite levels distinguishing healthy from diseased donors.

9 SUMMARY

This article has provided an overview of the common clinical analyses that may, in principle, be carried out by using IR spectroscopy, as well as pointing out some novel diagnostic tests that have emerged by combining IR spectroscopy with multivariate pattern recognition methods. In the case of analysis, it is clear that IR spectroscopy can meet the standards of accuracy that are required for a number of standard clinical serum and urine tests. More specialized tests such as amniotic fluid assays to assess fetal lung maturity may also be carried out, and other less common analyses, such as cerebrospinal fluid and interstitial fluid tests, can certainly be envisaged as being suited for IR spectroscopy.

IR spectroscopic analysis offers a number of practical advantages that make it a natural fit for high-volume applications of the type epitomized by serum and urine analysis, as well as for labor-intensive tests such as the amniotic fluid lecithin/sphingomyelin ratio. No reagents are required, there is generally no need to dilute very concentrated specimens (as may be required for certain other analytical methods), several analyses are available

simultaneously from a single IR spectrum, and – at least in the method using dried films – very little sample (microliters) is required. Time will tell whether these advantages will be exploited through the development of dedicated, IR-based clinical analyzers.

ACKNOWLEDGMENTS

Ms Sarah Low Ying is gratefully acknowledged for her assistance in the preparation of this article.

ABBREVIATIONS AND ACRONYMS

ATR	Attenuated Total Reflectance
FIR	Far-infrared
IR	Infrared
LDA	Linear Discriminant Analysis
MIR	Mid-infrared
MLR	Multiple-wavelength Linear Regression
NIR	Near-infrared
PCR	Principal Component Regression
PLS	Partial Least Squares
SEC	Standard Error of Calibration
SEP	Standard Error of Prediction
TLC	Thin-layer Chromatography

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